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Glucose Dehydrogenase

Technical field

The present invention relates to a glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme (PQQGDH), and its preparation and application to glucose quantification.

Background of the invention

Blood glucose concentration is an important marker for diabetes diagnosis. In addition, quantification of glucose concentration is used in monitoring the process of fermentative production using microorganisms. Conventionally, glucose quantification is performed by an enzymatic method using glucose oxidase (GOD) or glucose-6-phosphate dehydrogenase (G6PDH). However, the GOD method requires addition of catalase or peroxidase into the assay system to quantify hydrogen peroxide levels generated by oxidative reaction of glucose. G6PDH has been used for glucose quantification based on spectroscopy. This method involves the addition of coenzyme NAD(P) into the assay system.

Recently the application of PQQGDH, an enzyme which uses pyrroloquinoline quinone as a coenzyme is attracting attention, in place of the enzyme used in the existing glucose quantification method. PQQGDH is a glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme, and catalyzes the reaction of oxidizing glucose to produce gluconolactone.

Two types of PQQGDHs are known: membrane-bound and water-soluble. Membrane-bound PQQGDH is a single-peptide protein with an approximate molecular weight of 87 kDa, and is found in a wide variety of gram-negative bacteria. See, for example, J. Bacteriol. (1990) 172, 6308-6315, A. M. Cleton-Jansen et al. On the other hand, water-soluble PQQGDH

has been found in some strains of *Acinetobacter calcoaceticus* (Biosci. Biotech. Biochem. (1995), 59 (8), 1548-1555), and its structural gene has been cloned and its amino acid sequence determined (Mol. Gen. Genet. (1989), 217:430-436). Water-soluble PQQGDH derived from *A. calcoaceticus* is a homodimer enzyme with an approximate molecular weight of 50kDa. It shows little homology in primary structure with other PQQ enzymes.

Recently, the results of X-ray structural analysis of water-soluble PQQGDH were published and its conformation and active center were revealed (A. Oubrie, et al. (1999) J. Mol. Bio., 289, 319-333, A. Oubrie, et al. (1999) The EMBO Journal, 18 (19), 5187-5194, and A. Oubrie, et al. (1999), PNAS 96 (21), 11787-11791). These reports demonstrate that water-soluble PQQGDH is a β -propeller protein consisting of six W-motifs.

PQQGDH is expected to have potential in glucose assays, for example, as a recognition device of a glucose sensor, because it has highly oxidative activity towards glucose and does not require oxygen as an electron acceptor as it is complexed with a coenzyme. However, the low selectivity of PQQGDH for glucose was an obstacle to this use.

The object of this invention is to provide a modified water-soluble PQQGDH with a high selectivity for glucose.

Disclosure of the invention

As a result of extensive research to engineer conventional water-soluble PQQGDH to develop a PQQGDH which shows a higher selectivity for glucose and can be applied to clinical diagnosis and food analysis, the inventor successfully obtained an enzyme with higher selectivity by introducing amino acid mutations at certain regions of water-soluble PQQGDH.

The present invention provides a modified water-soluble glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme, wherein one or more amino acid residues of a wild type water-soluble glucose dehydrogenase are replaced with other amino acid residues and having high selectivity for glucose compared with the wild type water-soluble glucose dehydrogenase. The modified glucose dehydrogenase of the invention has higher glucose selectivity compared to the wild type water-soluble glucose dehydrogenase. Preferably, the modified glucose dehydrogenase of the invention has lower reactivity to lactose and maltose than to glucose compared to the wild type enzyme. More preferably, the modified glucose dehydrogenase of the invention has reactivity to lactose or maltose of less than 50%, even more preferably 40%, and most preferably 30% of the reactivity to glucose (100%).

One aspect of the invention provides a modified glucose dehydrogenase wherein one or more amino acid residues in a region of 186-206 amino acid of water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or in an equivalent region from other species are replaced with other amino acid residues (i.e., amino acid residues different from those found in a naturally occurring PQQ glucose dehydrogenase). Note that the amino acid numbering in this specification starts from the initiator methionine as the +1 position.

As used herein, the term "equivalent" with reference to the positions or regions of amino acid residues means that some amino acid residues or regions have an equivalent biological or biochemical function in two or more proteins which are structurally similar but not identical. For example, a certain region in water-soluble PQQGDH derived from organisms other than *Acinetobacter calcoaceticus* is said to be "equivalent to the region of amino acid residues 186-206 of water-soluble PQQGDH derived from *Acinetobacter*

calcoaceticus" when the amino acid sequence of such a region has a high similarity to the amino acid sequence in the 186-206 region of water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*, and the same function can be reasonably predicted based on the secondary structure of the relevant regions in the proteins. Additionally, the 7th amino acid residue of that region is said to be "an amino acid residue at the equivalent position to the amino acid residue 192 of water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*".

Preferably, in the modified glucose dehydrogenase of the invention, Gln192 or Leu193 of water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue in an equivalent position from other species are replaced with another amino acid residues.

In another aspect, the invention features a modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein Gln192 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with another amino acid residue. Preferably, Gln192 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with alanine, glycine, glutamic acid, leucine, phenylalanine, serine or aspartic acid.

In another aspect, the invention features a modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein both Gln192 and Asp167 of the amino acid sequence defined in SEQ ID NO: 1 are replaced with other amino acid residues. Preferably, Gln192 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with alanine, glycine, glutamic acid, leucine, phenylalanine, serine or aspartic acid. More preferably, Asp167 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with glutamic acid, and Gln192 is replaced with alanine, glycine, glutamic acid, leucine, phenylalanine, serine or aspartic acid.

In another aspect, the invention features a modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein Asp167 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with another amino acid residue, and Asn452 is replaced with another amino acid residue. Preferably, Asp167 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with glutamic acid. More preferably, Asp167 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with glutamic acid, and Asn452 is replaced with threonine.

In another aspect, the invention features a modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein Gln192 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with another amino acid residue, and Asn452 is replaced with another amino acid residue. Preferably, Gln192 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with alanine, glycine, glutamic acid, leucine, phenylalanine, serine or aspartic acid, and Asn452 is replaced with another amino acid residue. More preferably, Gln192 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with alanine, glycine, glutamic acid, leucine, phenylalanine, serine or aspartic acid, and Asn452 is replaced with threonine.

In another aspect, the invention features a modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein Leu193 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with another amino acid residue. Preferably, Leu193 of the amino acid sequence defined in SEQ ID NO: 1 is replaced with alanine, glycine, methionine, tryptophan or lysine.

In another aspect, the modified glucose dehydrogenase of the invention comprises the amino acid sequence:

Gly-Arg-Asn-Xaa1-Xaa2-Ala-Tyr-Leu

wherein Xaa1 and Xaa2 are independently any amino acid residues, provided that when Xaa1 is Gln, then Xaa2 is not Leu. Preferably, Xaa1 is Ala, Gly, Glu, Leu, Phe, Ser or Asp, and Xaa2 is Ala or Gly.

The invention also provides a gene coding for the modified glucose dehydrogenase of the invention, a vector comprising the gene of the invention and a transformant comprising the vector, as well as a glucose assay kit and a glucose sensor comprising the modified glucose dehydrogenase of the invention.

Since the enzyme protein of the modified glucose dehydrogenase of the invention shows high selectivity and high oxidization activity to glucose, it can be applied to highly specific and sensitive measurement of glucose.

Brief explanations of drawings

Fig.1 shows a structure of pGB2 plasmid used to construct mutant genes encoding modified PQQGDHs of the present invention.

Fig.2 shows a method of constructing mutant genes encoding modified PQQGDHs of the present invention.

Fig.3 is a graph showing substrate concentration dependency of the activity of the modified PQQGDHs of the present invention.

Detailed explanations of the invention

Structure of modified PQQGDH

In a preferred modified glucose dehydrogenase of the invention, one or more amino acid residues in the 186-206 amino acid region of water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or in an equivalent region from other species are replaced with other amino acid residues. Preferably, Gln192 of the amino acid sequence defined in SEQ

ID NO: 1 is replaced with alanine or glycine, and/or Leu193 is replaced with alanine, glycine, methionine, tryptophan or lysine.

In another aspect of the modified PQQGDH of the invention, in addition to the modifications as described above, Asp167 of the amino acid sequence defined in SEQ ID NO: 1 is also replaced with another amino acid, preferably with glutamic acid. Also preferably, in the modified PQQGDH of the present invention, in addition to the modifications as described above, Asn452 of the amino acid sequence defined in SEQ ID NO: 1 is also replaced with another amino acid, preferably with threonine. Involvement of Asp167 and Asn452 in recognition and binding of substrate by PQQGDH is described in Japanese Patent Public Disclosure Nos. 2001-346587 and 2001-197888, respectively. In general, however, no prediction can be made regarding the changes of substrate selectivity and enzyme activity which may be caused by simultaneously altering the amino acid residues in different domains. In some cases the enzyme activity will be completely abolished. Therefore, it was a surprising discovery in the present invention that improved selectivity for glucose can be achieved by introducing double mutations.

In another aspect, the modified glucose dehydrogenase of the present invention comprises the amino acid sequence: Gly-Arg-Asn-Xaa1-Xaa2-Ala-Tyr-Leu wherein Xaa1 and Xaa2 are independently any amino acid residues, provided that when Xaa1 is Gln, then Xaa2 is not Leu. Preferably, Xaa1 is Ala, Gly, Glu, Leu, Phe, Ser or Asn, and Xaa2 is Ala or Gly.

Preparation method of modified PQQGDH

The sequence of the gene encoding wild type water-soluble PQQGDH derived from *Acinetobacter*

calcoaceticus is defined in SEQ ID NO:2. Genes encoding modified PQQGDHs of the present invention can be constructed by replacing the nucleotide sequences encoding certain amino acids of the wild type water-soluble PQQGDH with the nucleotide sequences encoding the amino acids to be replaced. A wide range of methods for site-specific mutagenesis have been elaborated in the art, as described in, for example, Sambrook et al., "Molecular cloning; A Laboratory Manual", second edition, 1989, Cold Spring Harbor Laboratory Press, New York.

The mutant gene obtained in this manner is inserted into an expression vector (such as a plasmid) and transformed into an appropriate host (such as *E.coli*). A wide variety of host-vector systems have been developed in the art to express exogenous proteins. For example, bacteria, yeast, and cultured cells can be used as hosts.

As long as its glucose dehydrogenase activity is retained, the modified PQQGDH of the invention can further contain deletion, substitution or addition of other amino acid residues. A wide range of methods for site-specific substitution are available in the art.

Moreover, those skilled in the art can determine a region in a water-soluble PQQGDH derived from other bacteria which is equivalent to the region of the amino acid residues 186-206 of the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* by comparing the array of the primary structure of the proteins, or by comparing the secondary structures predicted from the primary structures of the enzymes. Thus, additional modified glucose dehydrogenases with improved glucose selectivity can be obtained by substituting amino acid residues in this region with another amino acid residues. Such modified glucose dehydrogenases are also within the scope of the present invention.

After culturing the transformants expressing modified PQQGDH, obtained as described above, the cells are collected by centrifugation and then crushed by French press, or the periplasmic enzymes may be released into the medium by osmotic shock. After ultracentrifugation, water soluble fractions containing PQQGDH can be obtained. Alternatively, expressed PQQGDH can be secreted into the culture by using an appropriate host-vector system. The water soluble fraction thus obtained is then purified by ion-exchange chromatography, affinity chromatography or HPLC, to obtain the modified PQQGDH of the invention.

Measurement of enzyme activity

The PQQGDH of the present invention catalyzes oxidation of glucose to produce gluconolactone using PQQ as a coenzyme. The enzyme activity can be quantified by color-developing reaction of a redox dye to measure the amount of PQQ reduced with glucose oxidation by PQQGDH. Example of color-developing reagents include PMS (Phenazine methosulfate), DCIP (2, 6-dichlorophenolindophenol), potassium ferricyanide, and ferrocene.

Glucose selectivity

The glucose selectivity of the present invention can be evaluated by measuring relative enzyme activity with respect to the activity for glucose by using a variety of sugars such as 2-deoxy-D-glucose, mannose, allose, 3-o-methyl-D-glucose, galactose, xylose, lactose, and maltose as a substrate.

The modified PQQGDH of the present invention shows improved glucose selectivity compared with wild type enzyme. Especially it has high reactivity to glucose compared with that to maltose. Therefore, the assay kit and enzyme sensor

prepared using the modified enzyme of the invention will exhibit high selectivity for glucose, and will have advantages in detecting glucose with high sensitivity even in samples containing variety of sugars.

Glucose assay kit

The present invention also provides a glucose assay kit containing the modified PQQGDH of the invention. The glucose assay kit of the invention may contain a sufficient quantity of the modified PQQGDH to carry out at least one assay. Besides modified PQQGDH, the kit may typically comprise buffers required for assay, a mediator, a standard solution of glucose to generate a calibration curve, and instructions for use. The modified PQQGDH can be supplied in a variety of forms, for example, as freeze-dried reagent or appropriate stock solutions. Preferably, the modified PQQGDH of the present invention may be supplied in the form of a holoenzyme, but can be supplied in the form of apoenzyme and converted into a holoenzyme before use.

Glucose sensor

The present invention also provides a glucose sensor containing the modified PQQGDH of the invention. Carbon, gold, or platinum may be used as an electrode, and the enzyme of the present invention is immobilized on the electrode. Immobilization methods includes, for example, methods using cross-linking reagents, inclusion into a macromolecular matrix, coating with dialysis membrane, methods using photo-crosslinking polymer, electric conductive polymer, and redox polymer. The enzyme can also be immobilized in a polymer or adsorbed on the electrode together with an electron mediator, such as ferrocene or its derivative. Combinations of the above may also be used. Preferably, the modified PQQGDH

of the present invention is immobilized on the electrode in the form of a holoenzyme, but can also be immobilized in the form of apoenzyme and PQQ is supplied as another layer or in solution. Typically, the modified PQQGDH of the present invention is immobilized on the electrode using glutaraldehyde, then free functional moieties of glutaraldehyde are blocked by treatment with a reagent having amine groups.

Measurement of glucose concentration is carried out as described below. Buffer, PQQ, CaCl_2 , and a mediator are placed into a constant-temperature cell and are kept at a constant temperature. Potassium ferricyanide and phenazine methosulfate may be used as a mediator. An electrode in which the modified PQQGDH of the present invention is immobilized are used as a working electrode, together with a counter electrode (e.g., platinum) and a reference electrode (e.g., Ag/AgCl electrode). A constant voltage is applied to the carbon electrode. After the current reaches a constant value, a glucose-containing sample is added and the increase in the current is measured. The glucose concentration in the sample can be calculated using a calibration curve generated by standard concentration glucose solutions.

All patents and references cited in this specification are incorporated by reference. All the contents disclosed in the specifications and drawings of Japanese Patent Application Nos. 2003-71760 and 2002-196177, on which the application claims priority, are incorporated herein by reference.

The working examples described below further illustrate the invention without limiting the present invention.

Example 1

Construction of gene encoding modified PQQGDH enzyme

Mutagenesis was carried out based on the structural gene of PQQGDH derived from *Acinetobacter calcoaceticus* (SEQ ID NO:2). pGB2 plasmid was constructed by inserting the structural gene of PQQGDH derived from *Acinetobacter calcoaceticus* into the multi-cloning site of pTrc99A vector (Pharmacia) (Fig.1). The nucleotide sequence encoding Gln192 or Leu193 was replaced with the nucleotide sequence encoding alanine, glycine, methionine, tryptophan or lysine by standard method of site-directed mutagenesis. Also the nucleotide sequence encoding Asp167 and Asp452 was replaced with the nucleotide sequence encoding glutamic acid and glycine, respectively. Site specific mutagenesis was performed using the pGB2 plasmid as shown in Fig.2. The sequences of synthetic oligonucleotide target primers used for mutagenesis are shown in Table 1. In order to construct a mutant containing two mutations, two oligonucleotide target primers were used simultaneously for mutagenesis.

Table 1

Gln192Ala	5'-ata agc aag cgg gtt acg ccc-3'
Gln192Gly	5'-caa ata agc aag ccc gtt acg ccc ttg-3'
Gln192Leu	5'-caa ata agc aag cag gtt acg ccc ttg-3'
Gln192Phe	5'-caa ata agc aag aaa gtt acg ccc ttg-3'
Gln192Ser	5'-caa ata agc aag gct gtt acg ccc ttg-3'
Gln192Asn	5'-caa ata agc aag gtt gtt acg ccc ttg-3'
Gln192Asp	5'-caa ata agc aag atc gtt acg ccc ttg-3'
Gln192Glu	5'-caa ata agc aag ttc gtt acg ccc ttg-3'
Gln192Lys	5'-caa ata agc aag ttt gtt acg ccc ttg-3'
Leu193Ala	5'-caa ata agc agc ctg gtt acg-3'
Leu193Gly	5'-gaa caa ata agc acc ctg gtt acg ccc-3'

Leu193Met	5'-gaa caa ata agc cat ctg gtt acg ccc-3'
Leu193Trp	5'-gaa caa ata agc ttt ctg gtt acg ccc-3'
Leu193Lys	5'-gaa caa ata agc cca ctg gtt acg ccc-3'
Asp167Glu	5'-cc tga ctg atg ttc ttt tga tga agg-3'
Asn452Thr	5'-c atc ttt ttg gac agt tcc ggc agt at-3'

A template was prepared by inserting the KpnI-HindIII fragment containing part of the gene encoding PQQGDH derived from *Acinetobacter calcoaceticus* into pKF18k vector plasmid (TaKaRa). A mixture of template (50 fmol), selection primer (5 pmol) supplied in Mutan-Express Km kit, phosphorylated target primer (50 pmol), and the annealing buffer supplied in the kit (1/10 of total volume (20 μ l)) was prepared, and plasmid DNA was denatured to single-strand by heating at 100 °C for 3 minutes. The selection primer was designed for the reversion of double-amber mutation on the Kanamycin resistance gene of the pKF18k plasmid. Plasmid DNA was put on ice for 5 minutes for annealing of the primers. A complementary strand was synthesized by adding the following reagents: 3 μ l of extension buffer supplied in the kit, 1 μ l of T4 DNA ligase, 1 μ l of T4 DNA polymerase, and 5 μ l of sterilized water. *E.coli* BMH71-18mutS, a DNA mismatch repair deficient strain, was transformed with the synthesized DNA and cultured overnight with vigorous shaking to amplify the plasmid.

Then, the plasmid was extracted from the bacteria and transformed into *E.coli* MV1184, and the plasmid was extracted from the colonies. The sequence of the plasmid was determined to confirm successful introduction of the desired mutations. Kpn I-Hind III gene fragment encoding wild type PQQGDH on pGB2 plasmid was replaced with the fragment containing the mutation to construct a series of mutated PQQGDH genes.

Example 2

Preparation of modified enzyme

A gene encoding wild type or modified PQQGDH was inserted into the multi-cloning site of pTrc99A (Pharmacia), and the constructed plasmid was transformed into *E.coli* DH5 α . Transformants were cultured in 450 ml of L-broth containing 50 μ g/ml of ampicillin and 30 μ g/ml of chloramphenicol using a Sakaguchi flask at 37 °C with vigorous shaking, and then inoculated in 7 L of L-broth containing 1 mM CaCl₂ and 500 μ M PQQ. After three hours of cultivation, IPTG was added to a final concentration of 0.3 mM, and cultivation was continued for another 1.5 hours. The cells were collected by centrifugation (5000xg, 10 min, 4 °C) and washed with 0.85% NaCl twice. The cells were crushed with French press (110 MPa), and centrifuged twice (10000xg, 15 min, 4 °C) to remove the debris. The supernatant was ultracentrifuged (160,500xg (40,000 rpm), 90 min, 4 °C) to obtain a water-soluble fraction. This fraction was used in the subsequent experiments as a crude enzyme preparation.

Example 3

Measurement of enzyme activity

Each of the crude enzyme preparation of wild type PQQGDH and modified PQQGDHs obtained in Example 2 was converted to a holoenzyme in the presence of 1 μ M PQQ and 1 mM CaCl₂ for 1 hour or more. The solution was divided into aliquots of 187 μ l each, and mixed with 3 μ l of activation reagents (6 mM DCIPA 48 μ l, 600 mM PMS 8 μ l, 10 mM phosphate buffer pH 7.0 16 μ l) and 10 μ l of D-glucose of various concentrations to measure the enzyme activity.

Enzyme activity was measured in MOPS-NaOH buffer (pH7.0) containing PMS (phenazine methosulfate)-DCIP (2, 6-dichlorophenolindophenol). Changes in absorbance of DCIP was recorded with a spectrophotometer at 600 nm, and the reduction rate of absorbance was defined as the reaction rate of the enzyme. In this measurement, enzyme activity which reduced 1 μmol of DCIP in one minute was defined as 1 unit. The molar absorption coefficient of DCIP at pH 7.0 was 16.3 mM^{-1} .

K_m was calculated from the plots of substrate concentration vs enzyme activity. The results are shown in Table 2.

Table 2

	K_m value for glucose(mM)	V_{max} (U/mg)
Wild type	30	129
Gln192Ala	50	123
Gln192Gly	36	94
Leu193Ala	177	42
Leu193Gly	157	46
Leu193Met	98	176
Leu193Trp	25	17
Leu193Lys	41	36

Example 4

Evaluation of substrate specificity

Substrate specificity of the crude preparation of the modified enzymes was examined. Each of the crude enzyme preparation of wild type PQQGDH and modified PQQGDHs obtained in Example 2 was converted to a holoenzyme in the presence of 1 μM PQQ and 1 mM CaCl_2 for 1 hour or more. The solution

was divided into aliquots of 187 μ l each, and mixed with 3 μ l of activation reagents (6 mM DCIPA 48 μ l, 600 mM PMS 8 μ l, 10 mM phosphate buffer pH 7.0 16 μ l) and substrate. Solution of glucose or other sugars (400mM) was added as a substrate to a final concentration of 20 mM or 100mM, and the mixture was incubated for 30 minutes at room temperature. The enzyme activity was measured in the same manner as Example 3. The values were calculated as relative activity to glucose (100%). The results are shown in Table3-6.

Table 3

	Wild type	Gln192Ala	Gln192Gly	Leu193Ala	Leu193Gly
Substrate conc	20mM	20mM	20mM	20mM	20mM
Glucose	100(%)	100(%)	100(%)	100(%)	100(%)
Allose	45	29	34	50	39
3-O-m-glucose	82	80	101	66	60
Galactose	8	10	12	34	26
Maltose	49	20	24	39	30
Lactose	53	56	40	64	56
Cellobiose	85	138	85	84	71

Table 4

	Wild type	Gln192Ala	Gln192Gly	Leu193Ala	Leu193Gly
Substrate conc.	100mM	100mM	100mM	100mM	100mM
Glucose	100(%)	100(%)	100(%)	100(%)	100(%)
Allose	62	41	45	47	35
3-O-m- glucose	92	93	98	86	59
Galactose	8	6	19	25	17
Maltose	51	56	44	50	46
Lactose	51	56	44	50	46
Cellobiose	42	73	59	59	39

Table 6

	Wild type	Leu193Met	Leu193Trp	Leu193Lys
Substrate conc.	20mM	20mM	20mM	20mM
Glucose	100(%)	100(%)	100(%)	100(%)
Galactose	11	36	24	43
Xylose	7	17	6	8
Lactose	61	59	76	48
Maltose	61	39	17	31

In addition, the enzyme activity of the modified enzyme of the present invention carrying a double mutation was measured. The results are shown in Table7, 8. Each modified enzyme of the present invention showed higher reactivity to glucose than to maltose.

Table 7

	Asp167Glu/ Asn452Thr	Gln192Gly/ Asn452Thr
Substrate conc.	20mM	20mM
Glucose	100(%)	100(%)
Allose	2	32
3-O-m-glucose	4	98
Galactose	2	14
Maltose	2	46
Lactose	12	21

Table 8

	Wild type		Asp167Glu/ Leu192Ala		Asp167Glu/ Leu192Gly		Asp167 Glu/ Gln192 Leu		Asp167Glu/ Gln192Ser		Asp167Glu/ Gln192Asn		Asp167 Glu/ Gln192 Glu		Asp167 Glu/ Gln192 Lys	
	20mM	100mM	20mM	100mM	20mM	100mM	100mM	100mM	20mM	100mM	20mM	100mM	20mM	100mM	100mM	100mM
Substrate conc.																
Glucose	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)
2-deoxyglucose	0	5	0	0	0	0	0	0	0	0	2	5	1	8		
Mannose	7	9	1	0	0	0	0	0	0	0	2	9	3	11		
Allose	41	65	6	5	6	7	2	1	0	16	26	5	63			
3-O-m-glucose	80	97	2	4	6	8	2	0	0	9	14	6	35			
Galactose	8	6	5	7	1	4	21	3	0	10	12	9	70			
Xylose	5	8	0	1	0	0	5	0	0	3	9	2	9			
Lactose	58	59	58	61	50	43	57	61	43	49	44	54	90			
Maltose	67	55	11	11	1	5	22	6	3	42	40	16	49			
Cellobiose	85	44	-	107	215	130	195	240	131	-	61	-	74			

Example 5

Dependency on substrate concentration

Dependency on substrate concentration of modified enzymes of the present invention was examined. Each modified enzyme was converted to a holoenzyme in the presence of 1 μ M PQQ and 1 mM CaCl_2 for 1 hour or more. Following the method for measuring the enzyme activity described in Example 3, the changes in absorbance of DCIP were measured with a spectrophotometer at 600nm as an indicator. The results are shown in Figure 3. The modified PQQGDH of the present invention was saturated at higher concentration of glucose compared with the wild type. For both modified and wild type PQQGDHs, substrate inhibition was not observed up to the glucose concentration of 200mM, and K_{si} was observed to be 200mM or more.

Example 6

Purification of enzyme

Each of the crude enzyme preparation of wild type and Gln192Asp obtained in Example 2 was adsorbed in a cation exchange chromatography column filled with TSKgel CM-TOYOPEARL 650M (Toso Co.) and equilibrated with 10 mM phosphate buffer, pH 7.0. The column was washed with 750 ml of 10 mM phosphate buffer, pH 7.0 and the enzyme was eluted with 10 mM phosphate buffer, pH 7.0 containing from 0 M to 0.2 M NaCl. The flow rate was 5 mL/min. Fractions showing GDH activity were collected and dialyzed against 10 mM MOPS-NaOH buffer (pH 7.0) overnight. In this manner, modified PQQGDH protein was purified which exhibited a single band under electrophoresis. Enzyme activity for various substrates of the purified enzyme was measured. The results are shown in Tables 9-10.

Table 9

	wild type					Glu192Asp				
	Km (mM)	Vmax (U/mg)	kcat (sec ⁻¹)	kcat/Km (mM ⁻¹ ·sec ⁻¹)		Km (mM)	Vmax (U/mg)	kcat (sec ⁻¹)	kcat/Km (mM ⁻¹ ·sec ⁻¹)	
glucose	25.0	4610	3860	154(100%)		50.0	475	398	8.0(100%)	
allose	35.5	2997	2509	71(46%)		57.2	226	189	3.3(42%)	
3-O-m- glucose	28.7	3596	3011	105(68%)		64.4	310	260	4.0(51%)	
galactose	5.3	277	232	44(29%)		118. 9	137	115	1.0(12%)	
lactose	18.9	1982	1659	88(57%)		75.0	390	327	4.4(54%)	
maltose	26.0	2305	1930	74(48%)		95.8	77	64	0.7(8%)	

Table 10

Asp167Glu/Asn452Thr			
	Km (mM)	kcat (sec ⁻¹)	kcat/Km (mM ⁻¹ ·sec ⁻¹)
Glucose	48	1193	25(100%)
Allose	182	73	0.4(2%)
3-O-m-glucose	198	215	1.1(4%)
Galactose	145	89	0.6(2%)
Lactose	55	167	3(12%)
Maltose	147	65	0.4(2%)
Cellobiose	16	226	14(56%)

Example 7

Preparation of enzyme sensor and its evaluation

Twenty mg of carbon paste was added to 5 units of the modified enzyme Gln192Ala and freeze-dried. The mixture was applied on the surface of a carbon paste electrode filled with approximately 40 mg of carbon paste, and the electrode was polished on a filter paper. This electrode was treated with MOPS buffer (pH 7.0) containing 1% glutaraldehyde for 30 minutes at room temperature and then treated with MOPS buffer (pH 7.0) containing 20 mM lysine for 20 minutes at room temperature to block unreacted glutaraldehyde. The electrode was equilibrated in 10 mM MOPS buffer (pH 7.0) for one hour or more at room temperature, then stored at 4 °C.

The glucose concentration was measured using the glucose sensor thus prepared. Glucose concentration was quantified in the range from 0.1 mM to 5 mM by using the glucose sensor prepared with the modified PQQGDH of the invention.

Industrial Applicability

The modified water-soluble PQQGDH of the present invention has high glucose selectivity, thus is useful as a device of a sensor for measuring blood glucose level.